Comp. Biochem. Physiol. Vol. 86B, No. 3, pp. 613–621, 1987
Printed in Great Britain

0305-0491/87 $3.00 + 0.00
Pergamon Journals Ltd

CHITINOLYTIC ENZYMES FROM PUPAE OF THE RED FLOUR BEETLE, *TRIBOLIUM CASTANEUM**

KARL J. KRAMER† and HIROYUKI AOKI

US Grain Marketing Research Laboratory, Agricultural Research Service, US Department of Agriculture, Manhattan, Kansas 66502, USA and Department of Biochemistry, Kansas State University, Manhattan, Kansas 66506, USA

(*Received* 12 *May* 1986)

Abstract—1. β-*N*-acetylglucosaminidase and one of the several chitinases were purified from the supernatant fraction from homogenates of pupae of the red flour beetle, *Tribolium castaneum* Herbst, by ammonium sulfate fractionation, hydroxylapatite chromatography, anion exchange chromatography and gel filtration chromatography.

2. β-*N*-acetylglucosaminidase exhibited a dimeric structure with apparent subunit molecular weights of 7.3×10^4 and 6.4×10^4, whereas chitinase was a monomer with an apparent mol. wt of 7.7×10^4.

3. β-*N*-acetylglucosaminidase and chitinase were composed of approximately 1200 and 700 amino acid residues per molecule and exhibited isoelectric points of pH 4.9 and 4.7, respectively.

4. The k_{cat}/K_m value for β-*N*-acetylglucosaminidase hydrolyzing $\beta GlcNAc_2$ was $2.22 \times 10^4\ M^{-1}\ sec^{-1}$ while that for chitinase hydrolyzing glycol chitin was $6.4\ ml\ mg^{-1}\ sec^{-1}$.

5. The results demonstrate a chitinolytic enzyme system in *T. castaneum* composed of an endo-splitting chitinase and an exo-splitting β-*N*-acetylglucosaminidase.

INTRODUCTION

Chitin is a cellulose-like biopolymer consisting predominantly of unbranched chains of $\beta(1\rightarrow4)$-2-acetamido-2-deoxy-D-glucopyranoside residues. It is found in fungi, yeasts, marine invertebrates and arthropods, where it is a principal component in the exoskeleton. Chitin is periodically synthesized and degraded during various morphogenetic stages. Limited information is available on chitin degradation and the associated enzymes (Kramer *et al.*, 1985). At least two enzymes are involved. They are β-*N*-acetylglucosaminidase (EC 3.2.1.30) and chitinase (EC 3.2.1.14) which facilitate the depolymerization of polymer to monomer. In preceding papers (Koga *et al.*, 1982, 1983), we showed that two β-*N*-acetylglucosaminidases and three chitinases are present in the tobacco hornworm, *Manduca sexta*. As part of a comparative biochemical study of insect chitinolytic enzymes, we describe here the purification and characterization of chitinolytic enzymes found in the pupae of the red flour beetle, *Tribolium castaneum*.

MATERIALS AND METHODS

Insects

The wild-type Lab-S strain of *Tribolium castaneum* Herbst was from the culture maintained at the US Grain Marketing Research Laboratory. Insects were reared on whole wheat flour containing 5% (w/w) brewer's yeast. Pupae of mixed age and sex were used immediately or frozen at −80°C until used.

Chemicals

Unless otherwise noted, all chemicals were of highest purity commercially available. Glycol chitin was prepared from crab chitin by glycolation with ethylene oxide followed by acetylation with acetic anhydride (Senzu and Okimasu, 1950). *N*-Acetylglucosamine oligosaccharides were prepared from crab chitin by the method of Raftery *et al.* (1969). Intact chitin and colloidal chitin were prepared as described previously (Fukamizo and Kramer, 1985). Fractogel TSK DEAE-650S and Fractogel TSK HW-55S were obtained from MCB Manufacturing Chemists, Inc.

Protein assay

Protein was determined with the Bio-Rad assay method based on the Coomassie Brilliant Blue dye binding procedure of Bradford (1976). Bovine serum albumin (Sigma) served as the standard protein.

Enzyme extraction

Pupae (20–45 g) were homogenized with a Tekmar tissumizer® in 50 mM potassium phosphate pH 6.8 containing 10 μM phenylthiourea (PTU, an inhibitor of tyrosinase), 1 mM diisopropylphosphorofluoridate (DFP, an inhibitor of serine proteases) and 20% (w/v) sucrose (0.42 g tissue wet wt/ml) for 5 min. After centrifugation at 20,500 *g* for 40 min at 4°C, the particulate matter was homogenized and centrifuged a second time and the combined supernatants dialyzed overnight against 6 l of the potassium phosphate buffer. For microsome preparation, pupae were homogenized in 50 mM *N,N-bis*(2-hydroxyethyl)-2-aminoethane sulfonic acid (BES) pH 7.1 containing 10 μM PTU and 8%

*Contribution No. 86-324-J. Department of Biochemistry, Kansas Agricultural Experiment Station, Manhattan, Kansas 66506. Cooperative investigation between ARS, USDA and the Kansas Agricultural Experiment Station. Mention of a proprietary product does not imply approval of this product by the USDA to the exclusion of other products that may also be suitable. Supported in part by USDA competitive research grants 82-CRCR-1-1055 and 85-CRCR-1-1667.

†Send correspondence to KJK, USGMRL, 1515 College Avenue, Manhattan, KS 66502, USA.

(w/v) sucrose (0.15 g wet wt per ml) for 5 min at 0°C. After centrifugation at 10,500 *g* for 15 min at 4°C, the supernatant was collected and centrifuged at 103,000 *g* for 1 hr at 4°C. The microsomal pellet was suspended in 3 ml of BES buffer and centrifuged a second time at 103,000 *g*.

Ammonium sulfate fractionation

Solid ammonium sulfate was added to the crude extract to give 30% saturation. After centrifugation at 20,500 *g* for 40 min at 4°C, the supernatant was mixed with ammonium sulfate to give 75% saturation. The precipitate was collected, dissolved in the 50 mM potassium phosphate buffer pH 6.8, and dialyzed overnight against several litres of 5 mM potassium phosphate pH 6.8.

Hydroxylapatite column chromatography

The dialyzed protein solution after ammonium sulfate fractionation was adsorbed onto a hydroxylapatite column (6 × 50 cm) equilibrated with 10 mM potassium phosphate, pH 6.8 at 4°C. The column was eluted with a linear gradient formed from equal volumes of 10 and 150 mM potassium phosphate (pH 6.8), and then finally eluted with 250 mM buffer. The active fractions were combined, concentrated by ultrafiltration and adsorbed onto a second hydroxylapatite column (3 × 30 cm) at 4°C. The column was washed with 10 mM potassium phosphate, and eluted using a linear gradient from equal volumes of 10 and 100 mM potassium phosphate buffer (pH 6.8).

DEAE-Fractogel column chromatography

The sample from hydroxylapatite chromatography was placed on a DEAE-Fractogel 650S column (3 × 35 cm) equilibrated with 10 mM potassium phosphate (pH 6.5). The column was subjected to a linear gradient formed with equal volumes of 10 and 100 mM potassium phosphate buffer (pH 6.5). Active fractions were combined and concentrated by ultrafiltration.

Gel-filtration chromatography

Samples from the anion-exchange chromatographic step were placed on a Fractogel HW-55S column (1.5 × 120 cm) equilibrated with 50 mM potassium phosphate (pH 6.8) and eluted with the same buffer. The β-*N*-acetylglucosaminidase fractions were also subjected to gel-filtration on Sephacryl S-300 column (2 × 172 cm) in 50 mM potassium phosphate (pH 6.8).

Electrophoresis

Electrophoresis was performed in polyacrylamide slab gels at pH 8.5 to assess purity and molecular weight under denaturing conditions in 0.1% SDS buffer (Weber *et al.*, 1972) and under nondenaturing conditions at pH 8.5 according to Davis (1964). The standard proteins and their apparent molecular weights were phosphorylase (9.2×10^4), bovine serum albumin (6.6×10^4), ovalbumin (4.5×10^4), carbonic anhydrase (3.1×10^4), soybean trypsin inhibitor (2.1×10^4) and lysozyme (1.4×10^4).

Isoelectrofocusing

A 4:1 mixture of pH 4–6.5 and 3–10 Pharmacia Pharmalytes was added to a bed formed with polyacrylamide gel. After focusing for 3 hr at 200 V, the gel was either stained with Coomassie Brilliant Blue G-250 or sectioned into 0.3 cm wide fractions for enzyme assay.

Ouchterlony procedure

Antisera raised against *M. sexta* chitinolytic enzymes were used to test for cross immunoreactivity with *Tribolium* enzymes (Koga *et al.*, 1983a, b). Ouchterlony gels were prepared in 1% (w/v) agarose, 50 mM sodium chloride and 0.01% thimerosal. Protein and antiserum were placed in wells and allowed to incubate overnight at 30°C.

Enzyme assay and kinetics

β-*N*-acetylglucosaminidase was assayed for pNpβGlcNAc hydrolytic activity by monitoring pNp production at 337 nm and for βGlcNAc oligosaccharide hydrolytic activity by high performance liquid chromatography (Koga *et al.*, 1982). Chitinase was assayed for glycol chitin hydrolytic activity by following the production of reducing end groups colorimetrically with the potassium ferriferrocyanide reagent at 420 nm (Imoto and Yagishita, 1971) and also for intact, colloidal or chitin oligosaccharide hydrolytic activity by HPLC (Koga *et al.*, 1983). Kinetic parameters were determined by Lineweaver–Burk plots (Koga *et al.*, 1982). Errors in individual determinations were $< 10\%$. Lytic activity for *Micrococcus lysodeikticus* cell walls was measured turbidimetrically (Neuberger and Wilson, 1967).

Hydrolysis of proteins and amino acid analysis

Purified enzymes were hydrolyzed in 4 N methanesulfonic acid with 0.2% (w/v) tryptamine according to Simpson *et al.* (1976) and in 6 N HCl containing 0.1% phenol according to Moore and Stein (1963). The amino acid composition was determined as described by Koga *et al.* (1982).

RESULTS

Purification of β-N-acetylglucosaminidase and chitinase

β-*N*-acetylglucosaminidase and chitinase activites were detected both in the microsomal and supernatant fractions from the homogenized crude extracts of pupae of *T. castaneum* but to different extents. Activities of β-*N*-acetylglucosaminidase and chitinase in the microsomal fraction were only about one-tenth of those present in the supernatant fraction. Thus, the chitinolytic enzymes of *T. casteneum* occur primarily as soluble proteins and the supernatant fraction of the pupal homogenate was used as the enzyme source.

β-*N*-acetylglucosaminidase and chitinase were purified from pupal homogenates of *T. castaneum* by ammonium sulfate fractionation, hydroxylapatite chromatography, DEAE anion-exchange chromatography and gel filtration chromatography. β-*N*-Acetylglucosaminidase was present as a single species, whereas chitinase exhibited multiple enzyme forms during chromatography. In the first hydroxylapatite chromatography, the main peaks of both β-*N*-acetylglucosaminidase(I) and chitinase(II) were eluted at 40 mM phosphate while a second peak of chitinolytic activity was observed at 150 mM phosphate (Fig. 1). The second peak was very heterogeneous and consisted of two or more chitinases. Only the major enzymes eluting at 40 mM phosphate were studied further. In DEAE-Fractogel chromatography, chitinase was eluted at 40 mM phosphate and β-*N*-acetylglucosaminidase at 70 mM phosphate (Fig. 2). After Fractogel HW-55S chromatography, the major peak of chitinase activity was pooled and utilized as a pure chitinase sample (Fig. 3). The final purifications were 306- and 302-fold with overall yields of 16 and 7% for β-*N*-acetylglucosaminidase and chitinase, respectively (Tables 1 and 2).

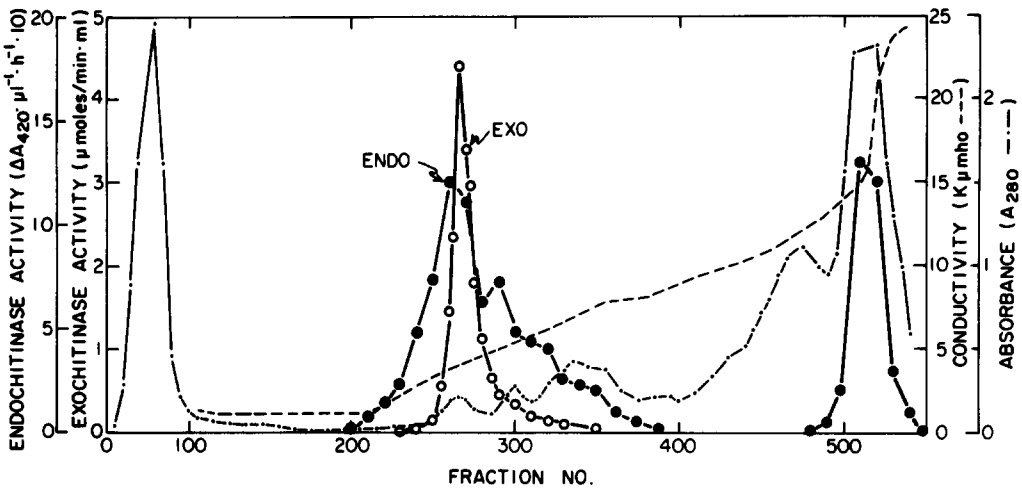

Fig. 1. First hydroxylapatite column chromatography of chitinolytic enzymes from pupae of *T. castaneum*. The dialyzed protein solution after ammonium sulfate fractionation was adsorbed onto a hydroxylapatite column (6 × 50 cm). Fractions 210–300 were pooled for further purification. 8 ml fractions were collected. Absorbance at 280 nm (—·—); conductivity (———); *β*-*N*-acetylglucosaminidase activity (○——○); chitinase activity (●——●).

Table 1. Purification of *β*-*N*-acetylglucosaminidase from *T. castaneum* pupae

Step	Total units (μmol min^{-1})	Total protein (mg)	Specific activity (μmol min^{-1} mg^{-1})	Overall yield*
Crude extract	352	3920	0.1	100
Ammonium sulfate precipitation	486	1620	0.3	98 (2)
Hydroxylapatite chromatography	First 465	64.6	7.2	87 (72)
	Second 319	38.5	8.3	59 (83)
Anion-exchange chromatography	293	18.7	15.7	55 (157)
Gel filtration	First 139	5.1	27.3	26 (273)
	Second 86	2.8	30.6	16 (306)

*Overall purification listed in parentheses.

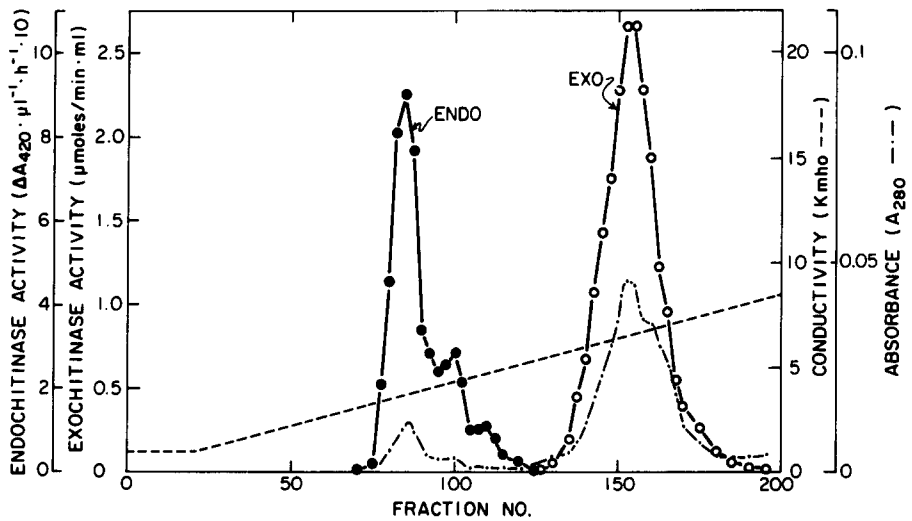

Fig. 2. DEAE-Fractogel column chromatography of chitinolytic enzymes from the second hydroxylapatite column step. The sample from second hydroxylapatite chromatography was placed on a DEAE-Fractogel 650S column (3 × 35 cm). Fractions 76–112 and 138–175 were pooled and used as chitinase and *β*-*N*-acetylglucosaminidase fractions for further steps, respectively. Phenol 8 ml fractions were collected. Absorbance at 280 nm (—·—); conductivity (———); *β*-*N*-acetylglucosaminidase activity (○——○); chitinase activity (●——●).

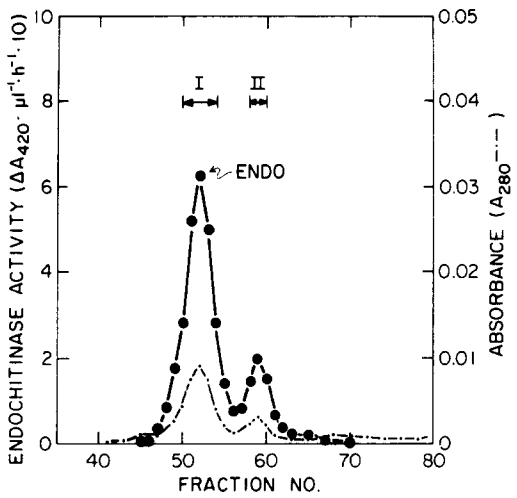

Fig. 3. Fractogel HW 55S gel filtration of *T. castaneum* chitinase. The chitinase sample from DEAE-Fractogel column chromatography was placed on a Fractogel HW 555 column (1.5 × 120 cm). The major peak chitinase activity (Fraction I, 50–54) was pooled and utilized as a pure chitinase sample. Absorbance at 280 nm (–··–); chitinase activity (●——●).

Homogeneity and molecular weight

Gel filtration of β-*N*-acetylglucosaminidase on Fractogel HW-55S and Sephacryl S-300 at pH 6.8 yielded a single symmetrical peak of activity coincident with absorbance at 280 nm (data not shown). Based on a comparison with elution volumes of standard proteins, the apparent molecular weight of β-*N*-acetylglucosaminidase was estimated to be 1.35×10^5 by gel filtration on Fractogel H-55S and 1.38×10^5 by chromatography on Sephacryl S-300. The enzyme migrated as a single protein during electrophoresis at pH 8.5 under nondenaturing conditions in 9% polyacrylamide. When subjected to polyacrylamide gel electrophoresis under denaturing conditions in the presence of sodium dodecyl sulfate, β-*N*-acetylglucosaminidase appeared as two bands both with or without 2-mercaptoethanol added to the sample. The apparent molecular weights of the two subunits were 7.3×10^4 and 6.4×10^4. Thus, β-*N*-acetylglucosaminidase appears to be a dimer composed of two nonidentical subunits. The apparent molecular weight of chitinase was estimated to be 7.8×10^4 by gel filtration on Fractogel TSK HW-55S, 7.6×10^4 by chromatography on Sephacryl S-300 and 7.7×10^4 by sodium dodecyl sulfate polyacylamide electrophoresis.

Amino acid analysis

The amino acid compositions of *T. castaneum* *N*-acetylglucosaminidase and chitinase are given in Table 3. Glutamic acid (or glutamine) and aspartic acid (or asparagine) were the two most abundant residues in both of the enzymes. The two enzymes exhibited rather similar amino acid compositions on a mole percentage basis, differing significantly only in levels of tryptophan, lysine, alanine and proline. β-*N*-acetylglucosaminidase and chitinase were composed of approx. 1200 residues and 700 residues, respectively. The molar extinction coefficients of β-*N*-acetylglucosaminidase and chitinase at 280 nm were calculated to be 2.19×10^5 and 7.37×10^4, respectively.

Table 3. Amino acid compositions of β-*N*-acetylglucosaminidase and chitinase from *T. castaneum*

Amino acid	Mole per cent (Residues per molecule)* β-*N*-acetylglucosaminidase	Chitinase
Asx	11.4 ± 1.9 (140)	10.6 ± 1.8 (74)
Glx	13.2 ± 1.0 (163)	13.0 ± 0.1 (91)
Ser	3.0 ± 0.2 (37)	3.8 ± 0.9 (27)
His	2.5 ± 0.2 (31)	2.1 ± 0.1 (15)
Thr	3.4 ± 0.9 (41)	3.1 ± 0.8 (21)
Gly	8.9 ± 0.6 (109)	8.0 ± 0.2 (56)
Ala	8.9 ± 0.3 (110)	9.7 ± 0.4 (68)
Tyr	3.3 ± 0.6 (40)	3.5 ± 0.4 (24)
Arg	1.6 ± 0.6 (20)	2.8 ± 0.3 (19)
Val	6.3 ± 0.2 (78)	6.7 ± 0.1 (47)
Met	1.9 ± 0.1 (23)	1.6 ± 0.5 (11)
Ile	5.1 ± 0.2 (63)	5.4 ± 0.1 (38)
Trp	3.2 ± 0.1 (40)	1.5 ± 0.2 (11)
Leu	8.7 ± 0.2 (107)	8.5 ± 0.2 (59)
Phe	3.4 ± 0.8 (42)	2.5 ± 0.1 (17)
Lys	7.1 ± 0.9 (87)	8.5 ± 0.2 (59)
Pro	6.0 ± 0.3 (74)	7.0 ± 0.5 (49)
Cys	2.1 ± 0.2 (26)	1.8 ± 0.3 (13)
Total residues	(1231)	(699)
Molecular weight	7.7×10^4	13.7×10^4

*Mean values from two analyses hydrolyzed for 24 hr in 4 *N* methanesulfonic acid with 0.2% (w/v) tryptamine and two analyses hydrolyzed for 24 hr in 6 *N* HCl containing 0.1% phenol.

Table 2. Purification of chitinase from *T. castaneum* pupae

Step	Total units (× 10^3)*	Total protein (mg)	Specific activity (Unit mg^{-1} × 10^3)	Overall yields†
Crude extract	161	3920	0.04	100 (1)
Ammonium sulfate precipitation	113	1620	0.07	70 (2)
Hydroxylapatite chromatography	First 38	65	0.59	24 (14)
	Second 24	38.5	0.62	15 (15)
Anion-exchange chromatography	19.5	1.9	10.3	12 (251)
Gel filtration	11.2	0.9	12.4	7 (302)

*Unit = 0.02 A at 420 nm after 1 hr at 32°C using 0.5 mg ml^{-1} glycol chitin in 50 mM sodium phosphate pH 6.4.
†Overall purification listed in parentheses.

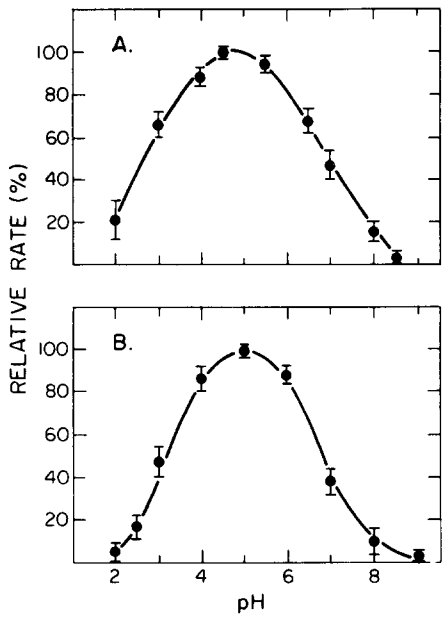

Fig. 4. Effect of pH on the hydrolysis of pNpβGlcNAc (A) and $\beta GlcNAc_2$ (B) by *T. castaneum* β-*N*-acetylglucosaminidase. A: The enzyme (0.7 nM) was incubated with pNpβGlcNAc (0.1 mM) in universal buffer of pH 2.0 to 8.5 at 25°C. Mean values from three determinations ±SE. B: The enzyme (0.25 nM) was mixed with dimer (0.24 mM) at 25°C. Reaction was quenched after 20 min by addition of dilute H_3PO_4 to pH 2.5. Mean values from four determinations ±SE.

Isoelectric focusing

Isoelectrofocusing of β-*N*-acetylglucosaminidase and chitinase in an ampholyte-containing polyacrylamide gel yielded single peaks of enzyme activity which coincided with Coomassie Brilliant blue G-250 stained bands. The isoelectric points (pI) of β-*N*-acetylglucosaminidase and chitinase were estimated to be pH 4.9 and 4.7, respectively.

pH dependence

The effect of pH on β-*N*-acetylglucosaminidase activity was examined using pNpβGlcNAc and the disaccharide β(1→4) $GlcNAc_2$ as substrates. Glycol chitin and the tetrasaccharide, β(1→4) $GlcNAc_4$, were used to study the pH dependence of chitinase. The pH-activity curves for β-*N*-acetylglucosaminidase degrading either of its substrates were relatively similar, and exhibited single pH optima between pH 4.5–5 and relatively symmetrical shapes (Fig. 4A, B). Apparently an ionized acidic group ($pK \sim 3$) and a protonated basic group ($pK \sim 7$) participate in the mechanism of β-*N*-acetylglucosaminidase catalysis.

The optimum pH for chitinase catalyzed hydrolysis of glycol chitin was about pH 8 (Fig. 5A). Chitinase exhibited a rather atypical profile with glycol chitin that suggests the existence of a second pH optimum as has been observed previously for *Manduca sexta* chitinase (Koga *et al.*, 1983). The optimum pH for tetramer hydrolysis by *T. castaneum* chitinase was about pH 6 (Fig. 5B). Fifty per cent of maximum activity occurred at approx. pH 4 on the acid side and at pH 9 on the alkaline side. This result suggested that an ionized acidic group and one or more protonated basic groups are required for enzymatic activity.

Kinetic parameters and substrate specificity

Table 4 shows the kinetic parameters for hydrolysis of pNpβGlcNAc and two chitin oligosaccharides by *T. castaneum* β-*N*-acetylglucosaminidase. At high substrate concentrations (>0.3 mM for pNpβGlcNAc and >0.4 mM for oligosaccharides), substrate inhibition was observed with β-*N*-acetylglucosaminidase. The kinetic constants were obtained at substrate concentrations in the range of 0.01–0.3 mM and 0.05–0.4 mM, respectively, where there was no deviation from linearity in Lineweaver–Burk plots. Products derived from the incubation of oligosaccharide with glucosaminidase were analyzed by HPLC. In the time courses for oligosaccharide hydrolysis, the initial products were monomer together with the saccharide reduced in size by one monomeric unit, a pattern typical of an exoenzyme mechanism. Although β-*N*-acetylglucosaminidase cleaved the small oligosaccharides rapidly, the k_{cat} value for pNpβGlcNAc is over 40 times larger than those of the chitin oligosaccharides. The smallest K_m value is also that of pNpβGlcNAc. As a result, the k_{cat}/K_m value for pNpβGlcNAc is more than two orders of magnitude greater than those for the chitin oligomers. These results showed that β-*N*-acetylglucosaminidase prefers smaller

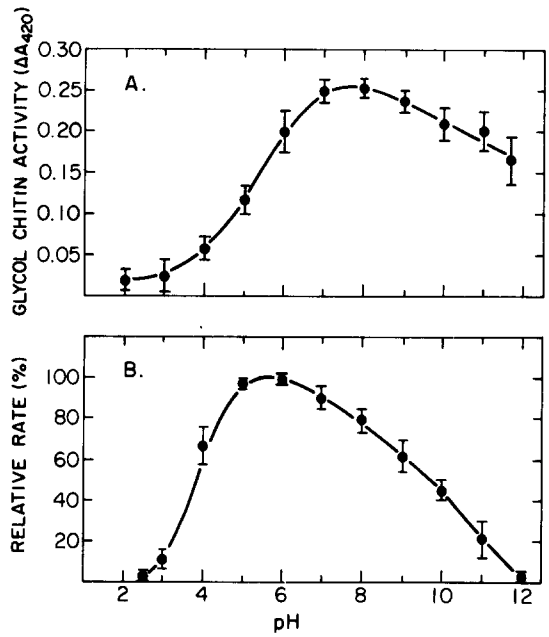

Fig. 5. Effect of pH on the degradation of glycol chitin (A) and the hydrolysis of $\beta GlcNAc_4$ (B) by *T. castaneum* chitinase. A: Enzyme (68 nM) was mixed with glycol chitin (0.33 mg/ml) in universal buffer (pH 2.0 to 12.0) for 1 hr at 32°C. Mean values from four determinations ±SE. B: Enzyme (9.1 nM) was mixed with tetramer (0.1 mM) at 25°C. Reaction was quenched after 20 min by addition of dilute H_3PO_4 to pH 2.5. Mean values for four determinations ±SE.

Table 4. Kinetic parameters for β-*N*-acetylglucosaminidase from *T. castaneum**

Substrate	k_{cat} (sec^{-1})	K_m ($M \times 10^3$)	k_{cat}/K_m ($sec\ M^{-1}\ 10^{-4}$)
pNpβGlcNAc	320 ± 25	0.071 ± 0.005	451
$\beta GlcNAc_2$	8.0 ± 1.0	0.363 ± 0.044	2.20
$\beta GlcNAc_3$	3.4 ± 1.0	0.216 ± 0.101	1.57

*$S_o = 10^{-5}$–10^{-3} M. $E_o = 2.8 \times 10^{-10}$ M. Mean values ± SEM $n = 4$.

chitin oligosaccharide substrates over larger ones and nitrophenylated GlcNAc over *N,N'*-diacetylchitobiose.

Substrate inhibition of *T. castaneum* chitinase was observed with both glycol chitin and chitin oligosaccharides. Inhibition caused by the former substrate was less severe than that caused by the latter such that Lineweaver–Burk plots could be used to determine a k_{cat} value of 1.80 ± 0.25 sec^{-1} and K_m value of 0.28 ± 0.06 mg ml^{-1} for glycol chitin. For the oligosaccharides k_{cat} and K_m values were estimated to be about 50 sec^{-1} and 0.1 mM, respectively. A comparison of the relative initial rates and cleavage patterns of chitin oligosaccharides and glycol chitin substrates by *T. castaneum* chitinase provided information about substrate susceptibility (Table 5). The hydrolytic rates increased as the length of substrate increased. No cleavage of disaccharide occurred and glycol chitin was degraded at a rate 10-fold or more faster than the *N*-acetylglucosamine oligomers. Cleavage patterns of oligosaccharides are those expected from an endo cleaving enzyme except that an exoenzyme activity was expressed with *N,N',N''*-triacetyl chitotriose as substrate. These results demonstrate that chitinase prefers larger substrates over smaller ones and is a true depolymerase. The active site of chitinase is probably rather large and consists of several binding subsites.

Neither enzyme exhibited activity towards *p*-nitrophenyl-2-acetamide-2-deoxy-α-glucopyranoside or *Micrococcus lysodeikticus* cell walls, the latter being a typical substrate for lysozyme. Chitinase was inactive toward *p*-nitrophenyl-2-acetamide-2-deoxy-β-D-glucopyranoside. Although chitinase showed an exoenzyme activity with *N,N',N''*-triacetylchitotriose as substrate, it exhibited the same endocleavage pattern with larger substrates as chitinase isolated from *M. sexta* (Koga *et al.*, 1983).

When *M. sexta* larval cuticular chitin or crab chitin were incubated at pH 7 with β-*N*-acetylglucosaminidase and chitinase of *T. castaneum*, they were almost completely depolymerized to 2-acetamido-2-deoxy-*D*-glucopyranoside (>90%). Products from colloidal and intact chitins were analyzed by HPLC. The mixture of β-*N*-acetylglucosaminidase and chitinase generated one major product *N*-acetylglucosamine and a minor unknown product probably a monoacetylated *N*-acetylchitobiose derivative detected in a previous study of *M. sexta* chitinolytic enzymes (Fukamizo *et al.*, 1986). Chitinase alone liberated *N,N'*-diacetylchitobiose as the major product, together with lesser amounts of *N*-acetylglucosamine, *N,N',N''*-triacetylchitotriose and the unknown compound. β-*N*-acetylglucosaminidase alone liberated only GlcNAc in 5–9% yield relative to the dual enzyme system.

Immunological relationship

In Ouchterlony double diffusion experiments antisera to the *M. sexta* β-*N*-acetylglucosaminidase and chitinase failed to crossreact with the heterologous enzymes from *T. castaneum* even at 10-fold higher protein concentrations than were necessary for homologous immunoprecipitations (Koga *et al.*, 1983b).

DISCUSSION

The present investigation was designed to accomplish the purification and characterization of *T. castaneum* β-*N*-acetylglucosaminidase and chitinase which would allow a comparison of those chitinolytic enzymes to similar enzymes from other sources. The molecular weight of *T. castaneum* β-*N*-acetylglucosaminidase was 1.37×10^5. This value is similar to the enzymes from the silkworm, *Bombyx mori* (1.25×10^5, Kimura, 1976), mold (1.4×10^5, Mega *et al.*, 1970) and shellfish (1.36×10^5, Phizackerley and Bannister, 1974), but different from the enzymes from the tobacco hornworm, *M. sexta* (6.0×10^4, Dziadik-Turner *et al.*, 1981) and from bacteria (7.5×10^4, Berkeley *et al.*, 1973). The *T. castaneum* enzyme exhibited a dimeric structure like that of β-*N*-acetylglucosaminidase from *B. mori*. While the silkworm enzyme was probably composed of identical subunits, the beetle glucosaminidase consisted of two distinct subunits. In polyacrylamide gel electrophoresis under denaturing conditions, *T. cas-*

Table 5. Relative rates of substrate hydrolysis by *T. castaneum* chitinase*

Substrate	Velocity (pmoles sec^{-1})	Cleavage pattern
$\beta GlcNAc_2$	0	X_1-X_2
$\beta GlcNAc_3$	15.1 ± 1.8 [6]†	X_1-X_2-X_3
$\beta GlcNAc_4$	17.7 ± 1.5 [7]	X_1-X_2-X_3-X_4
$\beta GlcNAc_5$	20.9 ± 2.1 [9]	X_1-X_2-X_3-X_4-X_5
$\beta GlcNAc_6$	23.1 ± 1.6 [10]	X_1-X_2-X_3-X_4-X_5-X_6
Glycol chitin	242 ± 19 [100]	X_1-X_2-X_3- -X_{n-1}-X_n

*$E_o = 9.04 \times 10^{-9}$ M, oligosaccharide $S_o = 1 \times 10^{-4}$ M, glycol chitin $S_o = 0.02$–0.2 mg ml^{-1}. Mean values ± SEM $n = 4$ to 6.

†Number in brackets gives relative rate of hydrolysis of all substrates by chitinase.

taneum glucosaminidase migrated as two protein bands with or without 2-mercaptoethanol in the sample buffer. These results suggested that there are two distinct subunits which are held together not by disulfide bonds but by noncovalent interactions.

The apparent molecular weight of *T. castaneum* chitinase was 7.7×10^4. The enzyme exhibited multiple forms during chromatography, but the major chitinase purified to homogeneity coincided in size with the largest of three chitinolytic enzyme species (7.5×10^4) from *M. sexta* (Koga *et al.*, 1983). It is smaller than one of the chitinases found in *Musca domestica* ($MW_{app} = 1.2 \times 10^5$, Singh and Vardanis, 1984) but larger than those present in *Cupiennius salei* ($MW_{app} = 4.8 \times 10^4$, Mommsen, 1980), *Drosophila hydei* ($MW_{app} = 4 \times 10^4$, Spindler, 1976), *Helix pomatia* ($MW_{app} \cong 2 \times 10^4$, Lundblad *et al.*, 1976) and *Stomoxys calcitrans* ($MW_{app} \sim 4.8 \times 10^4$, Chen *et al.*, 1981). Most of the chitinases appear to be monomeric proteins.

The pI value of *T. castaneum* β-*N*-acetylglucosaminidase was estimated to be 4.9. This value is similar to β-*N*-acetylglucosaminidases from *M. sexta* (Dziadik-Turner *et al.*, 1981), plants (4.9 and 4.65, Bouquelet and Spik, 1978) and mold (4.4, Jones and Kosman, 1980). The pI of *T. castaneum* chitinase was 4.7; The acidic value resembles those of other insect chitinases (Chen *et al.*, 1982, Koga *et al.*, 1983a), but it is different from wheat germ and *Streptomyces* chitinases that have slightly alkaline isoelectric points (Molano *et al.*, 1979; Charpentier and Percheron, 1983).

The pH activity profile for *T. castaneum* chitinase measured using glycol chitin and chitin tetraoligosaccharide as substrates showed maximum activity at pH ~ 8 and pH 6, respectively. These profiles were similar to those obtained for *M. sexta* chitinase (Koga *et al.*, 1983a). This unusual kinetic behavior is probably due to substrates that vary in either polymer chain length or degree of deacetylation.

The pH optima of β-*N*-acetylglucosaminidase for pNpβGlcNAc and chitin disaccharide $\beta(1\rightarrow4)$-$GlcNAc_2$ was between pH 4.5–5. These values are typical of many other β-*N*-acetylglucosaminidases such as those from the tobacco hornworm (Dziadik-Turner *et al.*, 1981), nematode (Bedi *et al.*, 1984) and mushroom (Sone and Misaki, 1978).

Amino acid analysis of *T. castaneum* β-*N*-acetylglucosaminidase showed a high mole percent of Glx and Asx (13.2 and 11.4%, respectively), and a relative low proportion of basic amino acids. The amino acid composition is similar to compositions reported for enzymes from other sources such as the nematode (Bedi *et al.*, 1984), mold (Mega *et al.*, 1970) and tobacco hornworm (Dziadik-Turner *et al.*, 1981). Although the values of Asp/Asn and Glu/Gln of β-*N*-acetylglucosaminidase were not measured individually, the low pI value of the enzyme suggests a relatively high proportion of Asp and Glu. The amino acid composition of chitinase was more similar to that of endochitinase I from *M. sexta* (Koga *et al.*, 1983) than that of endo-β-*N*-acetylglucosaminidase H from *Streptomyces grieus* (Robbins *et al.*, 1984). It is interesting to note that the amino acid compositions of *T. castaneum* β-*N*-acetylglucosaminidase and chitinase were very similar with ~38% nonpolar amino acids and similar proportion of acidic and basic residues. The apparent molecular weights of the subunits of β-*N*-acetylglucosaminidase were similar to that of chitinase. These results suggested that the two types of enzymes may have envolved from the same ancestral protein. However, there appears to be no apparent immunological relationship between the *T. castaneum* enzymes or related ones from the tobacco hornworm.

Two distinct β-*N*-acetylglucosaminidases were isolated and characterized from the tobacco hornworm (Dziadik-Turner *et al.*, 1981; Koga *et al.*, 1982). One of the enzymes was detected in larval and pupal hemolymph and the other in molting fluid, integument and pupal hemolymph. Red flour beetle β-*N*-acetylglucosaminidase is more like the former enzyme in that both exhibit a greater catalytic efficiency toward the nitrophenylated substrate than toward the chitin oligosaccharides. Flour beetle chitinase appears to be very similar to *M. sexta* molting fluid chitinase and probably has a relatively large active site which consists of several binding subsites. The catalytic action pattern of *T. castaneum* chitinase is essentially identical to those exhibited by enzymes from the tobacco hornworm (Fukamizo and Kramer, 1985a,b), fungi (Ohtakera and Mitsutoni, 1982; Beldman *et al.*, 1985), yeast (Barrett-Bee and Hamilton, 1984), bacteria (Young *et al.*, 1985a,b) and also cellulase from a mollusk (Anzai *et al.*, 1984). All are endo-type enzymes that exhibit random hydrolysis patterns during heterogeneous catalysis of polymeric substrates.

From kinetic studies it was shown that β-*N*-acetylglucosaminidase from *T. castaneum* pupae prefers smaller substrates such as $\beta(1\rightarrow4)GlcNAc_2$ over larger ones while chitinase prefers larger substrates such as $\beta(1\rightarrow4)GlcNAc_6$ and glycol chitin. The degradation of chitin is initiated by chitinase hydrolyzing native insoluble chitin ($GlcNAc_m$) to generate both soluble and insoluble intermediates by a random attack mechanism (Fukamizo and Kramer, 1985a,b). After several hydrolytic steps, relatively small soluble oligosaccharides such as $\beta(1\rightarrow4)$-$GlcNAc_{2\text{-}6}$ are generated. Penta- and hexasaccharide are examples of oligosaccharides that are further digested by chitinase to di- and trisaccharide. For the most part β-*N*-acetylglucosaminidase acts only on those small oligosaccharides and releases *N*-acetylglucosamine as the ultimate end-product.

Most of the *T. castaneum* chitinolytic activity is associated with the supernatant fraction of pupal homogenates. Only 6% of the chitinase and 10% of the β-*N*-acetylglucosaminidase activity in *T. castaneum* pupae is associated with microsomes. Fungal enzymes are distributed similarly with only minor amounts of activity detected in particulate fractions (Humphreys and Gooday, 1984). However zymogenic forms of chitinase are present in microsomal fractions of fungi.

Little information is available concerning the hormonal and other regulatory mechanisms for insect chitinolytic enzymes (Kramer *et al.*, 1985). Preliminary evidence suggests that inactive forms of chitinolytic enzymes or inhibitors of them may exist in *T. castaneum*. When crude pupal homogenates

were incubated with catalytic amounts of trypsin or chymotrypsin, both chitinase and β-*N*-acetylglucosaminidase activities were increased by as much as 40% (Aoki and Kramer, unpublished data). Calcium ion (5 mM) also elevated chitinase levels in crude homogenates. None of these effects were observed with purified enzyme preparations. A possibility exists that a calcium requiring proteolytic enzyme may activate zymogen forms of these enzymes. Molting fluid proteolytic enzymes digest cuticular protein in old cuticle (Katzenellenbogen and Kafatos, 1971). Whether those enzymes interact with chitinolytic enzymes is unknown. Recently the DNA and primary amino acid sequences of *Streptomyces* endo-β-*N*-acetylglucosaminidase was reported and it was suggested that this enzyme is synthesized as a proenzyme or preproenzyme (Robbins *et al.*, 1984). The same type of enzyme found in insects that catalyzes chitin catabolism may also be regulated *in vivo* by a posttranslational process such as proteolysis. We are now studying the regulatory properties of insect chitinolytic enzymes including a search for zymogen forms.

Acknowledgements—We are grateful to Dr George Lookhart, US Grain Marketing Research Laboratory, for assistance with the amino acid analyses.

REFERENCES

Anzai H., Nisizawa K. and Matsuda K. (1984) Purification and characterization of a cellulase from *Dolabella auricularia*. *J. Biochem.* (*Tokyo*) **96,** 1381–1390.

Barrett-Bee K. and Hamilton M. (1984) The detection and analysis of chitinase activity from the yeast forms of *Candida albicans*. *J. gen. Microbiol.* **130,** 1857–1861.

Bedi G. S., Shah R. H. and Bahl O. P. (1984) Studies on *Turbatrix aceti* β-*N*-acetylglucosaminidase. *Archs Biochem. Biophys.* **233,** 237–250.

Beldman G., Searle-Van Lecuwen M. F., Rombouts F. M. and Voragen F. G. J. (1985) The cellulase of *Trichoderma viride*. Purification, characterization and comparison of all detectable endoglucanases, exoglucamases and β-glucosidases. *Eur. J. Biochem.* **146,** 301–308.

Berkeleys R. C. W., Brewer S. J., Ortiz J. M. and Gillespie J. B. (1973) An exo-β-*N*-acetylglucosaminidase from *Bacillus subtilis* B: characterization. *Biochim. Biophys. Acta* **309,** 157–168.

Bouquelet S. and Spik G. (1978) Properties of four molecular forms of *N*-acetyl-β-D-hexosaminidase isolated from germinating seeds of fenugreek (*Trigonella foenum graecum*). *Eur. J. Biochem.* **84,** 551–559.

Bradford M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-die binding. *Analyt. Biochem.* **72,** 248–254.

Charpentier M. and Percheron F. (1983) The chitin-degrading enzyme system of a *Streptomyces* species. *Int. J. Biochem.* **15,** 289–292.

Chen A. C., Mayer R. T. and DeLoach J. R. (1982) Purification and characterization of chitinase from the stable fly, *Stomoxys calcitrans*. *Arch. Biochem. Biophys.* **216,** 314–321.

Davis B. J. (1964) Electrophoresis of proteins. *Ann. N.Y. Acad. Sci.* **121,** 404–427.

Dziadik-Turner C., Koga D., Mai M. S. and Kramer K. J. (1981) Purification and characterization of two β-*N*-acetylglucosaminidases from the tobacco hornworm, *Manduca sexta* (L.) *Arch. Biochem. Biophys.* **212,** 546–560.

Fukamizo T. and Kramer K. J. (1985a) Mechanism of chitin oligosaccharide hydrolysis by the binary enzyme chitinase system in insect moulting fluid. *Insect Biochem.* **15,** 1–7.

Fukamizo T. and Kramer K. J. (1985b) Mechanism of chitin hydrolysis by the binary chitinase system in insect moulting fluid. *Insect Biochem.* **15,** 141–145.

Fukamizo T., Kramer K. J., Mueller D. E., Schaefer J., Garbow J. and Jacob G. S. (1986) Analysis of chitin structure by nuclear magnetic resonance spectroscopy and chitinolytic enzyme digestion. *Arch. Biochem. Biophys.* **249,** 15–26.

Humphreys A. M. and Gooday G. W. (1984) Properties of chitinase activities from *Mucor mucedo*: evidence for a membrane-bound zymogenic form. *J. gen. Microbiol.* **130,** 1359–1366.

Imoto T. and Yagishita K. (1971) A simple activity measurement for lysozyme. *Agric. Biol. Chem.* **35,** 1154–1156.

Jones C. S. and Kosman D. J. (1980) Purification, properties, kinetics, and mechanism of β-*N*-acetylglucosaminidase from *Aspergillus niger*. *J. biol. Chem.* **255,** 11861–11869.

Katzenellenbogen B. S. and Kafatos F. C. (1971) Proteinases of silkworm moulting fluid: physical and catalytic properties. *J. Insect Physiol.* **17,** 775–800.

Kimura S. (1976) The chitinase system in the cuticle of the silkworm *Bombyx mori*. *Insect Biochem.* **6,** 479–482.

Koga D., Mai M. S., Dziadik-Turner C. and Kramer K. J. (1982) Kinetics and mechanism of exochitinase and β-*N*-acetylglucosaminidase from the tobacco hornworm, *Manduca sexta*. *Insect Biochem.* **12,** 493–499.

Koga D., Jilka J. and Kramer K. J. (1983a) Insect endochitinases: glycoproteins from moulting fluid, integument and pupal haemolymph. *Insect Biochem.* **13,** 295–305.

Koga D., Jilka J., Speirs R. D. and Kramer K. J. (1983b) Immunological relationships between β-*N*-acetylglucosaminidases from the tobacco hornworm, *Manduca sexta* (L.). *Insect Biochem.* **13,** 407–410.

Kramer K. J., Dziadik-Turner C. and Koga D. (1985) Chitin metabolism in insects. In *Comprehensive insect physiology, biochemistry and pharmacology: integument, respiration and circulation* (Edited by Kerkut G. A. and Gilbert L. I.), Vol. 3, Pergamon Press, New York. p. 75–115.

Lunblad G., Elander M. and Lind. J. (1976) Chitinase and β-*N*-acetylglucosaminidase in digestive juice of *Helix pomatia*. *Acta Chem. Scand.* **B30,** 889–894.

Mega T., Ikenaka T. and Matasushima Y. (1970) Studies on *N*-acetyl-β-D-glucosaminidase of *Aspergillus oryzae*. *J. Biochem.* **68,** 109–117.

Molano J., Polacheck I., Duran A. and Cabib E. (1979) An endochitinase from wheat germ: activity on nascent and preformed chitin. *J. biol. Chem.* **254,** 4901–4907.

Mommsen T. P. (1980) Chitinase and β-*N*-acetylglucosaminidase from the digestive fluid of the spider *Cupiennius salei*. *Biochim. Biophys. Acta* **612,** 361–372.

Moore S. and Stein W. H. (1963) Determination of amino acids in proteins. In *Methods in Enzymology* (Edited by Colowick S. P. and Kaplan N. D.), Vol. 6, pp. 819–831, Academic Press, New York.

Neuberger A. and Wilson B. M. (1967) Inhibition of lysozyme by derivatives of D-glucosamine. *Biochem. Biophys. Acta* **147,** 473–479.

Ohtakara A. and Mitsutomi M. (1982) Mode of hydrolysis of chitoligosaccharides with chitinase and chitobiase produced by *Pycnoporus cinnabarinus*. In *Chitin and Chitosan. Proc. Second Internat. Conf. Chitin and Chitosan* (Edited by Hirano S. and Tokura S.), Jap. Soc. Chitin and Chitosan. pp. 117–121.

Phizackerley P. J. R. and Bannister J. V. (1974) The characterization of *N*-acetylglucosaminidase from the limpet *Patella vulgata* (L.). *Biochim. Biophys. Acta* **362,** 129–135.

Robbins P. W., Trimble R. B., Wirth D. F., Hering C., Maley F., Maley G. F., Das R., Gibson B. W., Royal N. and Biemann K. (1984) Primary structure of the *Streptomyces* enzyme endo-β-*N*-acetyl-glucosaminidase H. *J. biol. Chem.* **259,** 7577–7583.

Raftery M. A., Rand-Meir T., Dahlquist F. W., Parsons S. M., Borders C. L., Wolcott R. G., Beranek W. and Jao L. (1969) Separation of glycosaminoglucan saccharide and glycoside mixtures by gel filtration. *Analyt. Biochem.* **30,** 427–431.

Senzu R. and Okimasu S. (1950) Preparation of glycol chitin. *Nippon Nogei Kagaku Kaishi* **23,** 432–437.

Simpson R. J., Neuberger M. R. and Liu T.-Y. (1976) Complete amino acid analysis of proteins from a single hydrolysate. *J. biol. Chem.* **251,** 1936–1940.

Singh G. J. P. and Vardanis A. (1984) Chitinases in the house fly, *Musca domestica*. Pattern of activity in the life cycle and preliminary characterization. *Insect Biochem.* **14,** 215–218.

Sone Y. and Misaki A. (1978) Purification and characterization of β-D-mannosidase and β-*N*-acetyl-D-hexosaminidase of *Tremella fuciformis*. *J. Biochem.* **83,** 1135–1144.

Spindler K. (1976) Initial characterization of chitinase and chitobiase from the integument of *Drosophila hydei*. *Insect Biochem.* **6,** 663–667.

Weber K., Pringle J. R. and Osborn M. (1972) Measurement of molecular weights by electrophoresis on SDS-acrylamide gel. In *Methods in Enzymology* (Edited by Hirs C. W. H. and Timasheff N.), Vol. 26, pp. 3–27, Academic Press. New York.

Young M. E., Bell R. L. and Carroad P. A. (1985a) Kinetics of chitinase production. I. Chitin hydrolysis. *Biotech. Bioengng* **27,** 769–775.

Young M. E., Bell R. L. and Carroad P. A. (1985a) Kinetics of chitinase production. II. Relationship between bacterial growth, chitin hydrolysis, and enzyme synthesis. *Biotech. Bioengng* **27,** 776–780.